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(71) Applicant *(for all designated States except US)*: MARICULTURA, INCORPORATED [US/US]; Post Office Drawer 565, Wrightsville Beach, NC 28480 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)* : LONG, Thomas, Veach, II [US/US]; 315-B Summer Rest Road, Wilmington, NC 28403 (US).

(74) Agent: BARBER, Lynn, E.; Olive & Olive, P.O. Box 2049, Durham, NC 27702 (US).

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(54) Title: MICROORGANISM PRODUCTION OF OMEGA-3 (N-3) LIPIDS

(57) Abstract

The invention comprises the use of obligately and facultatively marine eukaryotic microorganisms for the production of Omega-3 (n-3) fatty acids that may be used in food, cosmetic, and pharmaceutical products. In the invention the microorganisms are grown heterotrophically, harvested, and extracted for lipid products.

MICROORGANISM PRODUCTION OF OMEGA-3 (N-3) LIPIDS

Field of the Invention

This invention relates to fatty acid production from microorganisms. In particular, this invention relates to the use of obligately or facultatively marine eukaryotic microorganisms grown heterotrophically and the production of a group of highly polyunsaturated fatty acids known as Omega-3 or n-3 fatty acids.

Background Information

The biosynthesis of fats and oils by microbes such as yeasts, bacteria, molds, and algae is well established, and inventors have devised ways of optimizing the growth conditions for this biosynthesis. For example, yeasts or molds in mixed culture with bacteria and grown on mixtures of carbohydrates or hydrocarbons under aerobic fermentation conditions were found to produce numerous amino acids plus, in some cases, unspecified oil and fat. (U.S. Pat. No. 3,793,153). The disclosure of this reference and all others cited herein are hereby incorporated by reference. Yeasts such as Candida and Rhodotorula also produce single-cell protein and undifferentiated lipid from vegetable carbohydrates including starch (U.S. Pat. No. 4,230,806). Under aerobic conditions Candida tropicalis produces unsaturated dicarboxylic acids having 14 to 22 carbon atoms from unsaturated fatty acids or their esters (U.S. Pat. No. 4,474,882). Numerous yeasts dramatically increase their production of 1, 3-disaturated-2-unsaturated triglycerides, a predominant component of cacao butter, under high oxygen fermentation conditions in a growth medium containing one or more fatty acids having between 10 and 20 carbon atoms. (U.S. Pat. No. 4,485,173). Rhodococcus rhodochrous (formerly

Nocardia aurantia) synthesizes hydroxy-fatty acids and keto-fatty acids, such as 10-hydroxy-12-octadecenoic acid and 10-hydroxysteric acid, under aerobic conditions by hydration of unsaturated fatty acids such as linoleic and oleic acid, respectively. (U.S. Pat. No. 4,582,804). Methods have also been devised to increase oil production and recovery from halophilic algae such as Dunaliella by growing the cells photosynthetically in a saline solution with a growth promoting enzyme and using water insoluble solvent extraction. The oils obtained had a carbon and nitrogen content similar to crude oil and were apparently saturated fatty acids and wax esters. (U.S. Pat. No. 4,341,038).

Although the inventors of the cited patents and other investigators have determined ways to obtain and optimize fat and oil production from certain microorganisms, none of these methods produces polyunsaturated fatty acids, and, in particular, they do not yield the highly polyunsaturated fatty acids designated Omega-3 or n-3 fatty acids. The Omega-3 and Omega-6 fatty acids are the two principal classes of polyunsaturated fatty acids. The Omega-3 (or n-3) fatty acids have the first unsaturation (double bond) at the third carbon from the methyl (or Omega) end of the fatty acid, and the Omega-6 (or n-6) fatty acids have the first unsaturation at the sixth carbon from the methyl end of the molecule. The Omega-6 fatty acids, such as linoleic acid, are common components of vegetable oils, such as corn oil. The microbial production of linoleic acid by culturing fungi of the Pellicularia genus has been elaborated (U.S. Pat. No. 4,281,064).

The Omega-3 fatty acids are prominent components of some fish oils, although they are not synthesized by the fish but, instead, appear to be absorbed from their diets. These highly polyunsaturated fatty acids have been shown effective in humans in preventing or remedying cardiovascular disease and a number of auto-immune diseases, such as arthritis. They

appear to be an essential nutrient. Although an earlier fermentation process of the prokaryotic Streptomyces organisms (U.S. Pat. No. 3,127,315) produced a hypocholesterolemic agent referred to as M-850, it is clear from the extraction procedures used that this agent is not related to the Omega-3 fatty acids or their action.

The focus of medical research on the fatty acids has been principally on two of the Omega-3 fatty acids, eicosapentaenoic acid (EPA: a 20-carbon fatty acid having 5 unsaturations) and docosahexaenoic acid (DHA: a 22-carbon fatty acid having 6 unsaturations), although others of this class may prove to be important. It is widely recognized that the principal dietary sources of these chemical moieties for fish are photosynthetic algae and microalgae. The use of the marine microalga, Chlorella minutissima, to produce Omega-3 fatty acids photoautotrophically has been the subject of recent patents (U.S. Pat. No. 4,615,839 and Jap. Pat. Discl. No. Sho-61-63624). A process for preparation of eicosapentaenoic acid from linolenic acid using enzymes from microalgae and macroalgae also has been described (Jap. Pat. Discl. No. Sho-61-31092).

In contrast, no method has been described heretofore to produce Omega-3 fatty acids from microbes that are grown heterotrophically, using as a nutrient a sugar, carbohydrate, or other source of "pre-formed" carbon. Although a process for cultivating the freshwater microalga Chlorella mixotrophically on lower fatty acids to aid in disposing of organic wastes (U.S. Pat. No. 3,444,647) was developed, no product description is provided, and this species does not produce Omega-3 fatty acids.

Summary of Invention

This invention comprises use of certain heterotrophic and autotrophic eukaryotes grown heterotrophically to produce

lipid that contains Omega-3 fatty acids as prominent constituents, and in use of this lipid or its constitutents in nutritional supplements; in foods for humans and other animals, including aquacultured species; and in drugs and pharmaceuticals. No prokaryote is known to synthesize Omega-3 fatty acids. Eukaryotic synthesis based on autotrophic growth of photosynthetic microalgae presents problems of light and nutrient provision that impair economic production. The inventor has determined that obligately or facultatively marine eukaryotes, including but not limited to fungi, thraustochytrids, microalgae (in particular, diatoms and dinoflagellates), and yeasts produce high concentrations of docosahexaenoic acid and eicosapentaenoic acid when grown under heterotrophic growth conditions. Obligately freshwater (aquatic) heterotrophic eukaryotes do not appear to produce these chemicals.

Conventional sources of Omega-3 fatty acids are oils from such fish as salmon, anchovies, sardines and menhaden. These fish oils in the unrefined form contain cholesterol. Depending on the environment and diet of the fish, these fish oils may also contain heavy metals and synthetic organic chemicals such as polychlorinated biphenyls (PCB's), polybrominated biphenyls (PBB's), dieldrin and aldrin. In addition to these disadvantages, the fish oils have an unpleasant fishy odor and taste. It is a further advantage of this invention to provide lipids containing Omega-3 fatty acids that are not contaminated with heavy metals or undesirable synthetic organic chemicals. The invention also provides Omega-3 fatty acid products that do not have a strong fishy odor and taste.

Detailed Description of the Invention

This invention represents that selected heterotrophic eukaryotes that are either halo-tolerant or halophilic, when cultured, cultivated, or fermented in a salt-containing medium

or one containing natural or artificial seawater, will produce Omega-3 fatty acids, including but not limited to EPA and DHA, as a significant percentage of total lipid. Furthermore, the use of such microorganisms yields a lipid fraction that is useful as a nutritional supplement; as a food additive in margarines, cooking oils, salad dressings, baked products, infant nutritional formulae and adult enteral nutritional formulae; as a skin care product or cosmetic; as a drug or pharmaceutical; as a component of an intravenous, parenterally administered fluid; and as an animal or aquaculture feed or feed additive. The high levels of Omega-3 fatty acids produced by these marine eukaryotes grown heterotrophically are unique. Prokaryotic microorganisms generally do not produce these fatty acids. Although photosynthetic, autotrophic eukaryotes do synthesize the Omega-3 fatty acids, the rates of growth of these organisms and their production of the fatty acids under photosynthetic growth conditions are significantly less than when heterotrophic eukaryotes are cultivated heterotrophically. Specific heterotrophs appropriate for use in this invention include, but are not limited to: the thraustochytrids, Thraustochytrium roseum and T. aureum; the phycomycetes fungi, Pythium sp. and Schizochytrium aggregatum; the diatom, Nitzschia sp.; and the dinoflagellate, Crypthecodinium cohni. These species are maintained in the American Type Culture Collection and the algae culture collection of the University of Texas at Austin.

The Omega-3 fatty acids appear to be produced only by marine microorganisms or by halophilic or halo-tolerant species. Thus, in the preferred embodiment, either seawater, an artificial seawater, or other saline solution is used as the solvent in the culture medium. The complete medium is referred to as a saline culture medium. The carbon source in this saline culture medium may be a relatively simple carbohydrate source, for example, glucose, sucrose, mannose, or molasses, or, if slower cultivation is permitted, vegetable fibers such as grasses or bagasse.

After inoculation of the saline culture medium with the selected eukaryotic microorganisms, the medium is incubated under conditions favorable for heterotrophic growth of the microorganism. Extractions of the culture are accomplished by solvent extraction using a mixed organic solvent and standard techniques. This invention will be more clearly understood by references to the following illustrative examples, which are not to be construed as limiting the invention:

EXAMPLE 1

Pure cultures of the thraustochytrids, yeasts, fungi, or microalgae are inoculated into liquid media of successively larger volumes starting at 100 ml and staging up to larger cultures. A saline culture medium is prepared by mixing 1.0 g of glucose with 0.1 g yeast extract in 1000 ml of aged seawater. The amount of glucose can be increased to 5 g and the pH in the preferred embodiment is adjusted to between 7 and 7.5. The cultures are grown for two weeks at approximately 25 to 28° C. to facilitate heterotrophic growth. Growth vessels are either shaken with rotary shakers or magnetically stirred. The species may be grown either unilluminated or illuminated with moderate light, even though the organisms are growing heterotrophically. Harvesting is carried out by centrifugation or freeze drying.

These microorgansims may be extracted in the wet state directly after harvesting or in the freeze dried state, using a mixture of non-polar and polar organic solvents consisting of methanol, chloroform, and water in the proportions 2:1:0.8. The solvents are mixed with the microorganisms and allowed to stand for 1/2 to 3 hours. After this period, additional chloroform and water are added to yield a solvent ratio of 2:2:1.8 of methanol, chloroform, and water. The chloroform layer contains the total lipid fraction, which is comprised of a sufficiently high concentration of Omega-3 fatty acids to be useful in nutrition and medicine.

Example 2

The procedures followed in Example 1 are used, except that the saline culture medium is composed of 1 to 5 g glucose, 1 g yeast extract, and 1 g peptone in 1 liter of seawater. The pH is adjusted to between 7 and 7.5.

Example 3

The procedures followed in Example 1 are utilized, except that an artificial seawater base is prepared for this saline culture medium. This consists of 2.5 g NaCl, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.1 g KCl, 0.01 g KH_2PO_4, 0.02 g $CaCO_3$ and sufficient H_2SO_4 to dissolve the above compounds. To this solution, $(NH_4)_2 SO_4$ is added in the amount of 0.02 g, along with 0.2 with 0.2 g NaH-glutamate, 0.1 g Agar, 1.0 ug Thiamine-HCl, 0.1 ug Cyanocobalamin, 5.0 mg Na_2EDTA, and the trace metals 0.05 mg $FeSO_4 \cdot 7H_2O$, 0.02 mg $ZnSO_4 \cdot 7H_2O$, 0.01 mg $MnSO_4 \cdot H_2O$, 2.0 ug $CoSO_4 \cdot 7H_2O$, 0.2 ug $CuSO_4 \cdot 5H_2O$, 2.0 ug H_3BO_3, 2.0 ug $NaMoO_4 \cdot H_2O$ with sufficient distilled water to yield 100 ml of solution. To this is added 0.1 to 0.5 g glucose or other sugar, 0.01 g $NaHCO_3$, and the pH is adjusted to between 7 and 7.5.

Example 4

The media and procedures employed in any of the above examples are used except that the cultures are exposed to light of moderate intensity. Heterotrophic growth of certain marine eukaryotes, such as the thraustochytrids, is enhanced under such conditions.

Example 5

The media and procedures employed in any of the above examples are used except that additional limited quantities of available N and P (such as 0.085 g $NaNO_3$ and 0.012 g NaH_2PO_4) are added to the saline culture medium. Heterotrophic growth

of certain marine eukaryotes, such as the microalgae, is enhanced in this medium.

The samples harvested from these examples produce lipid fractions containing Omega-3 fatty acids. After extraction and esterification to form the methyl esters, gas chromatographic analyses show that the Omega-3 fatty acids may constitute as much as 10 to 50% of the total fatty acid fraction. They are generally contained in phospholipids, glycolipids, mono-, di-, or triglycerides, and sulfolipids, or as the free acids, but are not limited to these forms.

What is claimed is:

1. A process of using heterotrophically grown obligately or facultatively marine eukaryotic microorganisms as a source for the production of Omega-3 (n-3) fatty acids.

2. A process according to claim 1, wherein the marine eukaryotic microorganism is selected from the group consisting of thraustochytrids, lower fungi, yeasts, and microalgae.

3. A process for the production of Omega-3 (n-3) fatty acid products from obligately or facultatively marine eukaryotes, comprising the steps of:

(a) inoculating a saline culture medium containing a carbon source with marine eukaryotic microorgansims;

(b) incubating the inoculated saline culture medium under conditions conducive to heterotrophic growth of the marine eukaryotic microorganisms;

(c) harvesting the marine eukaryotic microorganisms from the saline culture medium; and

(d) extracting the lipid fraction from the harvested marine eukaryotic microorganisms.

4. A process according the claim 3, wherein the saline culture medium comprises seawater.

5. A process according to claim 4, wherein the carbon source comprises glucose.

6. A process according to claim 4, wherein the conditions conducive to heterotrophic growth comprise a pH of from 7

to 7.5 and a temperature of 25 to 28° C.

7. A process according to claim 3, wherein the saline culture medium comprises an artificial seawater base.

8. A process according to claim 7, wherein the carbon source comprises glucose.

9. A process according to claim 7, wherein the conditions conducive to heterotrophic growth comprise a pH of from 7 to 7.5 and a temperature of 25 to 28° C.

10. A process according to claim 3, wherein the conditions conducive to heterotrophic growth comprise exposure to light of moderate intensity.

11. A fatty acid product comprising an Omega-3 (n-3) fatty acid extracted from a heterotrophically grown, obligately or facultatively marine, eukaryotic microorganism culture.

12. The fatty acid product of claim 11, wherein the fatty acid product may be used as a nutritional additive to the diet of humans.

13. The fatty acid product of claim 11, wherein the fatty acid product may be used as a pharmaceutical product.

14. The fatty acid product of claim 11, wherein the fatty acid product may be used as a skin care or cosmetic product.

15. The fatty acid product of claim 11, wherein the fatty acid product may be used as an animal feed additive.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US88/02483

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): C12P 7/ 64; C09F 5/02; A61K 7/00
U.S. CL.: 435/134; 260/405.5; 426/60

II. FIELDS SEARCHED

Minimum Documentation Searched

Classification System	Classification Symbols
U.S.	435/134, 254, 257, 911, 946, 171, 255 426/60; 260/405.5; 514/844, 772

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched

Databases: Chemical Abstracts Services Online (File CA, 1967-1988; File Biosis 1969-1988 US Patent File (USPAT) . See attachment for search terms.

III. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	Journal of The Canadian Dietetic Association, (Toronto, Canada), Volume 143, issued April 1982, (ACKMAN, R.G.), "Polyunsaturated Fatty Acid Content of Kelp Tablets and Dulse", pages 150-154. See entire document.	12 and 13-15
X Y	Lancet (London, England), Volume 2, issued 15 July 1978, (DYERBERG ET AL). "Eicosopentaenoic Acid and Prevention of Thrombosis and Atherosclerosis?," pages 117-119. See entire document.	12-15 13

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
30 August 1988	14 NOV 1988
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Irene Marx IRENE MARX

III DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X / Y	Bulletin of the Japanese Society of Scientific Fisheries (Tokyo, Japan), Volume 46, issued 1980, (WATANABE ET AL.) "Relationship between Dietary Value of Brine Shrimp Artemia salina and their content of omega-3. Highly unsaturated Fatty acids", pages 35-41. See entire document.	15 / 12-13
Y	US, A, 4,661,343 (ZABOTTO) 28 April 1987, See columns 4 and 5 in particular.	14
X / Y	Journal of Protozoology (Athens, Georgia), Volume 17, issued 1970 (HARRINGTON ET AL.) "The Polyunsaturated Fatty Acids of Marine Dinoflagellates", pages 213-219. See entire document.	1-3, and 5-8 / 4-6, 9 and 10
X / Y	Comparative Biochemistry and Physiology, (Elmsford, New York), Volume 29, issued 1969, (ELLENBOGEN, B. ET AL,) "Polyunsaturated Fatty Acids of aquatic fungi: possible phylogenetic significance". pages 805-811. See entire document.	1-3, 7 and 10 / 4-6 and 8-9
X / Y	Journal of Experimental Botany (Oxford, England) Volume 25, issued August 1974 (OPUTE, F.I.), "Lipid and Fatty Acid Composition of Diatoms" pages 823-835. See entire document.	1-3, 7 and 10 / 4-6 and 8-9
Y	Mycologia (Bronx, New York), Volume 55, issued 1963 (GOLDSTEIN, S.) "Studies of a new species of Thraustochytrium that displays light stimulated growth", pages 799-811. See entire document.	1-10

Search Terms

Eicosapentaenoic

docosahexaenoic

cosmetic or skin care

polyunsaturated or unsaturated

animal (feed or food)

(omega-3 or n-3) fatty acid

lipid

halotolenant or halophilic

marine, salin?, salt

fungus, fungi, alga?

microbe, microorganism

diatom, dinoflagellate

ferment

heterotroph

thraustochytrid

phycomycetes

pythium

schizochytrium

Nitzschia